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13. ABSTRACT *(Maximum 200 Words)*

The goal of this study was to develop biomarkers of toxicant exposure, in rodent models, with a focus on lead a and the military jet fuel JP8. Our results demonstrated that these toxicants caused significant alterations in the levels of specific detoxication enzymes. These affected enzymes are members of the family of glutathione S-transferases (GSTs), enzymes which detoxify many environmental toxicants and drugs. Studies on lead effects on kidney found that large increases in these enzymes occurred at lead levels seen in the environment of exposed persons and they preceded pathobiological changes in kidney structure and function in lead-treated rats, suggested that changes in GSTs are a sensitive tissue marker of toxicant exposure. Studies on mechanisms demonstrated that the observed changes in GSTs reflected changes in gene expression, and contrary to prevailing dogma, did not result from oxidative stress. Inhalation exposure of JP8 was shown to affect other members of the GST family in the nervous system, with cerebellum and retina affected. These results suggest that these regions of the nervous system are targets of JP8 toxicity; this is of Air Force significance, since visual and motor functions are controlled by retina and cerebellum.

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FINAL TECHNICAL REPORT
AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
GRANT NO. F49620-96-1-0074
BIOMARKERS OF TOXICANT EXPOSURE

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CO-PRINCIPAL INVESTIGATOR: STEVEN E. KORNGUTH, Ph.D.
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MAY 1, 1999

1. OBJECTIVES: To characterize biomarkers of exposure to toxic levels of lead and also to the jet fuel JP8.

2. RESEARCH RESULTS

a) Background - This project was designed to develop exposure biomarkers for lead and JP8 toxicity. In research supported by our original AFOSR grant, we found that high dose lead administration caused dramatic increases in specific isoenzyme forms of the detoxication enzyme glutathione S-transferase (GST) in kidney. Increases in these GSTs were followed by pathobiological changes in renal architecture and decreased kidney function, suggesting that increases in kidney GSTs were an early warning sign of lead toxicity and were valid tissue biomarkers. These studies were made possible by our development of an analytical protocol which allowed for the quantitative analysis of fifteen GST isoenzymes, using high performance liquid chromatography (hplc). Our goals for the period covered by this report included the following:

(1) To determine the effects of lead exposure on the expression of GSTs in tissues other than kidney.

(2) To determine if the lead-related increases in GSTs were the result of increased transcription (gene expression).

(3) To determine if lead at low doses which mimic environmental exposure also cause changes in tissue GSTs.

(4) To test the prevailing hypothesis that lead effects on GST expression result from oxidative stress.

(5) To determine if inhalation exposure to the aviation jet fuel JP8 also causes changes in tissue GSTs.

Each of these goals has been attained and the results have either been published or, in the case of goal (5), submitted for publication.

b) Inorganic Lead Study

Experimental design - Adult Sprague-Dawley rats were given daily intraperitoneal (i. p.) injections of lead acetate; control rats were given equal volume injections of physiological saline.

Results

Dose-response study - A dose-response study of the effect of lead acetate administration on rat kidney glutathione S-transferase was performed, and the results demonstrate that increases

in GST activity are highly correlated to the lead dose and the concomitant blood lead levels. Sprague-Dawley rats were injected with doses of lead acetate ranging from 0.1 to 114 mg/kg (0.3 to 300 μmole/kg) for 3 consecutive days and sacrificed 24 hr later. Kidney GST activity, GST isoform HPLC profiles, blood lead analysis, serum clinical chemistry panels, and light microscopy were performed. Treatment with 1 mg/kg lead acetate corresponded to a blood lead level of 26 μg/dl and produced a significant increase in total GST activity which continued to increase with dose up to 38 mg/kg. This increase in GST activity paralleled the changes in blood lead levels. When the data sets of dose, weight loss, blood lead levels, and total GST activity were correlated with respect to each other. Individual GST isoforms exhibited different thresholds and maxima. rGSTP1 and rGSTM1 had thresholds of 1 mg/kg and 3.8 mg/kg respectively, very similar rates of increase with dose, and a maximum yield that was 450% above control at a dose of 38 mg/kg for both enzymes. rGSTA1 and rGSTA3 showed similar thresholds (1 mg/kg) and maximal fold increase (275%) but varied in the relative response to each dose. rGSTA2 and rGSTM2 had only modest increases beginning at a dose of 38 mg/kg. Minimal changes in rGSTA4, M3, and M6 and microscopic structural changes were detected only at the highest dose. A standard clinical chemistry panel was run on serum from each animal, and no significant changes were observed for any component, including, blood urea nitrogen (BUN), creatinine, glucose and γ-glutamyl transferase (GGT). **These results indicate that renal GST increases occur at lead levels which are environmentally significant, and at doses that precede any clinical serum or histological changes, and also suggest that GST can serve as highly sensitive biomarker of lead exposure.**

Lead mode of action study - The effect of acute exposure to lead acetate on the expression of glutathione S-transferase isoenzymes, level of glutathione, and amount of malonaldehyde in rat kidney and liver was determined. The purpose of this study was to determine if GSH depletion and oxidative stress are responsible for the increase in GST following lead exposure. In kidney rGSTM1, rGSTA2, and rGSTP1 all increase following i.p. injection of lead. The level of GSH is not effected 0.5 or 1 hour after lead exposure, but is increased 3, 6, 12, and 24 hours after a single injection of lead. MDA levels (a marker of lipid peroxidation) do not change in kidney. Therefore, we conclude that the increases in GST isoenzymes that occur in kidney following lead exposure are not dependent upon GSH depletion or oxidative stress. In liver, GSH depletion (61% of control 12 hours after lead treatment) and increased MDA production (2.5-fold increase 6 hours after lead exposure) occur, while rGSTM1 and rGSTA2 do not increase. Immunoperoxidase light microscopy and immunogold electron microscopy reveal that the increase in kidney GSTM1 and GSTP1 occurs in both the nuclei, cytoplasm, and microvilli of proximal tubules. Northern blot analysis of rGSTA2 and rGSTP1 showed that the mRNA increase of these two GSTs following lead exposure can be inhibited by actinomycin D, suggestion transcriptional induction. 2-Dimensional gel electrophoresis show not only induction of rGSTP1 in kidney after lead treatment, but also appearance of newly charged species of this isoenzyme. **This study demonstrates that acute lead exposure causes dramatic changes in the subcellular distribution and expression of GST isoenzymes, and that these changes are not a result of GSH depletion or oxidative stress.**

c. Organic lead study

Experimental design - Adult male Sprague-Dawley rats were given injections of triethyl lead chloride; control animals received injections of physiological saline.

Results - The effects of triethyl lead chloride (TEL) on the expression of glutathione S-transferases (GSTs) and NAD(P)H:quinone oxidoreductase (QR) in rat liver and kidney were determined. Fischer 344 rats were given one injection intraperitoneally of TEL. GST activity, GST isoform levels, mRNA levels of alpha class GST isoforms A1, A2, and A3 and activity of QR were determined. Treatment of rats with TEL caused a significant increase in GST activity in kidney. The levels of GSTs M1, P1, A3, A1, A2, and A4 were significantly elevated in kidney, while the level of GSTM2 was unchanged. The largest increase was a 3.2-fold increase in GSTM1. The levels of GSTA1, A2, and A3 mRNA in kidney also increased significantly after injection of TEL. In liver, TEL injection resulted in decreased GST activity; the level of hepatic GSTs M2, P1, M3, A1, A2, and A4 decreased significantly following the injection of TEL. The largest decrease was a 40 percent reduction of GST A1. In contrast, the level of liver GST A3 increased from day 4 through day 14 after injection of TEL. The levels of liver mRNAs coding for alpha class GSTs YA1, A2, and A3 were reduced 12 hours after injection of TEL. By 24 hours after TEL injection, GST A1 and A2 mRNA levels returned to basal level while A3 message increased to a level higher than controls. The activity of QR was elevated 1.5-fold in kidney and 2.7-fold in liver 14 days after the injection of TEL. This report demonstrates that administration of organic lead significantly affects GST expression and QR activity in a tissue-specific and isoform-specific manner. An unexpected observation was the onset of violent aggressive behavior in rats given triethyl lead. **These results indicate that the effects of TEL on GST expression and QR activity must involve more than a single promoter.**

c) JP8 Jet Fuel Study - Swiss-Webster mice and adult rats were exposed to JP8 aerosol in the AFOSR-supported Inhalation Toxicology Laboratory of Dr. Mark Witten, Departement of Pediatrics, University of Arizona Medical School.

Experimental design - In the inhalation toxicology study, animals were exposed to JP8 aerosol in the AFOSR-supported laboratory of Dr. Mark Witten, Department of Pediatrics, University of Arizona and tissue samples were analyzed in our facilities at the University of Wisconsin.

Results - Male mice were exposed to aerosolized jet fuel (JP8+100) at concentrations of 1000 mg/m^3 or 2500 mg/m^3, 1-10micron diameter particle size. Exposure was for one hour per day for seven days. The animals were then sacrificed and we examined the retina, brain regions, liver, lung and kidney to determine whether such treatment resulted in changes in the concentration or cell distribution of the enzyme glutathione S transferase (GST). The retina and cerebellum tissues were of particular interest because of the critical roles of proprioception and vision in Air Force personnel. and then sacrificed. The retina was studied

immunohistochemically while other brain regions, including cerebellum were studied immunohistochemically, by HPLC, and by GST enzyme activity. Other tissues were studied by HPLC. The immunohistochemical studies revealed that the JP8+100 affected the concentration of GSTs in the radial glial cells of cerebellum and retina as determined immunohistochemically. The major changes observed were the increased immunoreactivity of the anti-GSTM1 antisera with the Bergmann glial cells of cerebellum and Muller cells of retina. We also observed a decreased immunoreactivity of the cerebellar molecular layer with anti-GSTP. **These results are consistent with the functional changes in proprioception and light sensitivity observed in personnel who are exposed to high concentrations of JP8+100.**

3. PERSONNEL SUPPORTED

Frank L. Siegel, Ph.D., Principal Investigator
Steven E. Kornguth, Ph.D., Co-Principal Investigator
Lynda S. Wright, M.S., Med. Tech., Senior Researcher
Shelli A. Nelson, B.S., Research Specialist
Danniel A. Daggett Ph. D. (1997), Graduate Research Assistant (no salary from AFOSR)

4. PUBLICATIONS (1996-1999)

a) Published or In Press

McGuire, S., Daggett, D., Bostad, E., Schroeder, S., Siegel, F. And Kornguth, S. (1996) Cellular Localization of Glutathione S-Transferases in Retinas of Control and Lead-Treated Rats, Investigative Ophthalmology and Visual Science **37**, 833-842.

Daggett, D. A., Nuwaysir, E. F., Nelson, S. A., Wright, L. S., Kornguth, S. E. And Siegel, F. L. (1996) Effects of triethyl lead administration on the expression of glutathione S-transferase isoenzymes and quinone reductase in rat kidney and liver. Toxicology **117**, 61-71.

McGuire, S., Daggett, D., Bostad, E., Schroeder, S., Wright, L., Siegel, F. and Kornguth, S. (1997), Increased levels of glutathione transferases and appearance of novel alpha class isoenzymes in kidneys of mice exposed to mercuric chloride. Nephron **77**, 452-460.

Witzmann, F.A., Daggett, D.A., Fultz,C.D., Nelson, S.A., Wright, L.S., Kornguth, S.E. and Siegel, F. L. (1998) Glutathione S-transferases: Two-dimensional electrophoretic protein markers of lead exposure. Electrophoresis **19**, 1322-1335.

Witzmann, F. A., Fultz, C. D., Grant, R. A., Wright, L. S., Kornguth, S. E. and Siegel, F. L. (1998) Differential expression of cytosolic proteins in the rat kidney and cortex: preliminary proteomics. Electrophoresis **19**, 2491-2497.

Daggett, D. A., Oberley, T. D., Nelson, S. A., Wright, L. S., Kornguth, S. E. and Siegel, F. L. (1998) Effects of lead on rat kidney and liver: GST expression and oxidative stress. Toxicology **128**, 191-206.

Wright, L. S., Kornguth, S. E., Oberley, T. D. and Siegel, F. L. (1998) Effects of lead on glutathione S-transferase expression in rat kidney: a dose-response study. Toxicol. Sci. **46**, 254-259.

Witzmann, F. A., Fultz, C. D., Grant, R. A., Wright, L. S., Kornguth, S. E. and Siegel, F. L. (1999) Regional protein alterations in rat kidneys induced by lead exposure. Electrophoresis **20**, 943-951.

b) Submitted for Publication

Wright, L. S., McGuire, S., Bostad, E., Nelson, S. A., Daggett, D. A., Witten, M. L., Siegel, F. L. and Kornguth, S. E., Effects of jet fuel JP8+100 aerosol on glutathione S-transferase expression in retina and cerebellum of Swiss-Webster mice.

5. DISSERTATIONS

Daggett, D. A. (1997) Effects of Lead on the Expression of Glutathione S-Transferases, Ph.D. Dissertation, University of Wisconsin - Madison. Obtainable through the University of Wisconsin - Madison Memorial Library. All key results included in this dissertation have been published.

6. INTERACTIONS/TRANSITIONS

a) Invited presentations

Frank L. Siegel: Merck Lectureship, Memorial University, St. John's Newfoundland, May, 1997.

Steven E. Kornguth: Lectures to Institute for Advanced Technology, University of Texas at Austin, February, 1999.

Steven E. Kornguth: DARPA, Monterey, California, 1998.

Steven E. Kornguth: AFOSR JP8 Toxicology Conference, San Antonio, Texas, April, 1998

Steven E. Kornguth and Lynda S. Wright: AFOSR JP-8 Jet Fuel Toxicology Workshop, The University of Arizona, December, 1998.

b) Consulting for the Department of Defense

Steven E. Kornguth: Environmental Sciences Group, Brooks Air Force Base, San Antonio, Texas; Institute for Defense Analysis, Alexandria, Virginia.

APPENDIX

In a preliminary experiment to determine the neurochemical effects of JP8 exposure, adult rats were exposed to JP8 aerosol at a dose of 1000 mg/m^3 for either 7 or 14 days in the laboratory of Dr. Mark Witten, University of Arizona. We analyzed brain regions for catecholamines and serotonin neurotransmitters and their metabolites.

Neurotransmitter Levels after 7 and 14 day JP8 Aerosol Exposure

Neurotransmitter	**striatum control**	**7 day striatum**	**14 day striatum**
Norepinephrine	3.867 ± 0.677	2.002 ± 0.373	3.352 ± 0.34
Dopamine	5.046 ± 0.498	6.846 ± 0.816*	2.365 ± 1.885
DOPAC	23.47 ± 2.12	45.93 ± 6.22**	20.00 ± 2.74
HVA	1.922 ± 0.172	2.982 ± 0.539*	1.246 ± 0.191*
Serotonin	7.981 ± 0.931	4.949 ± 0.741*	5.607 ± 0.341*
5-HIAA	3.807 ± 0..172	5.041 ± 0.803	2.441 ± 0.172

Neurotransmitter	**cortex control**	**7 day cortex**	**14 day cortex**
Norepinephrine	2.178 ± 0.223	1.270 ± 0.129*	1.742 ± 0.168
Dopamine	0.802 ± 0.108	0.380 ± 0.050*	0.385 ± 0.310*
DOPAC	0.827 ± 0.188	0.525 ± 0.060	1.722 ± 0.258*
HVA	0.069 ± 0.010	0.038 ± 0.003	0.094 ± 0.011
Serotonin	4.357 ± 0.290	2.243 ± 0.394*	4.036 ± 0.594
5-HIAA	2.775 ± 0.406	1.964 ± 0.290	2.528 ± 0.284

Neurotransmitter	**cerebellum control**	**7 day cerebellum**	**14 day cerebellum**
Norepinephrine	2.251 ± 0.281	1.674 ± 0.162	1.731 ± 0.179
Dopamine	0.823 ± 0.062	0.619 ± 0.116	0.423 ± 0.052*
DOPAC	0.330 ± 0.379	0.341 ± 0.045	0.335 ± 0.056
HVA	0.068 ± 0.006	0.065 ± 0.007	0.060 ± 0.008
Serotonin	1.085 ± 0.146	0.741 ± 0.102	0.839 ± 0.120
5-HIAA	0.766 ± 0.052	0.662 ± 0.106	0.703 ± 0.128

Neurotransmitter	**hippocamp control**	**7 day hippocamp**	**14 day hippocamp**
Norepinephrine	2.112 ± 0.321	2.929 ± 0.504	2.657 ± 0.343
Dopamine	0.860 ± 0.210	0.660 ± 0.103	0.658 ± 0.143
DOPAC	2.246 ± 0.818	2.162 ± 0.455	3.038 ± 0.952
HVA	0.158 ± 0.039	0.133 ± 0.023	0.209 ± 0.026
Serotonin	4.433 ± 0.624	4.170 ± 0.669	5.144 ± 0.678
5-HIAA	3.467 ± 0.551	5.086 ± 0.740	3.988 ± 0.625

Neurotransmitter	midbrain control	7 day midbrain	14 day midbrain
Norepinephrine	4.284 ± 0.393	3.729 ± 0.309	3.412 ± 0.370
Dopamine	0.780 ± 0.100	0.405 ± 0.045	0.426 ± 0.072
DOPAC	2.579 ± 0.448	2.623 ± 0.303	2.153 ± 0.303
HVA	0.246 ± 0.062	0.133 ± 0.020	0.164 ± 0.033
Serotonin	7.396 ± 0.209	6.490 ± 0.370	5.780 ± 0.392*
5-HIAA	4.626 ± 0.305	4.389 ± 0.334	3.841 ± 0.426

Neurotransmitter	brain stem control	7 day brain stem	14 day brain stem
Norepinephrine	2.921 ±0.265	3.057 ± 0.428	2.600 ± 0.231
Dopamine	0.292 ± 0.035	0.129 ± 0.013	0.198 ± 0.032
DOPAC	0.913 ± 0.154	0.732 ± 0.112	0.584 ± 0.131
HVA	0.109 ± 0.010	0.089 ± 0.011	0.114 ± 0.013
Serotonin	4.759 ± 0.434	4.421 ± 0.634	4.709 ± 0.545
5-HIAA	2.640 ± 0.340	2.610 ± 0.333	2.707 ± 0.289

*- $p < 0.05$
**- $p < 0.01$

These preliminary results indicate that JP8 inhalation produces significant changes in the levels of brain neurotransmitters.